Kruppel-Like Factor 2 (KLF2) Regulates Endothelial Thrombotic Function


Abstract—The vascular endothelium maintains blood fluidity by inhibiting blood coagulation, inhibiting platelet aggregation, and promoting fibrinolysis. Endothelial cells lose these nonthrombogenic properties on exposure to proinflammatory stimuli. We recently identified the Kruppel-like factor KLF2 as a novel regulator of endothelial proinflammatory activation. Here it is found that KLF2 differentially regulates key factors involved in maintaining an antithrombotic endothelial surface. Overexpression of KLF2 strongly induced thrombomodulin (TM) and endothelial nitric oxide synthase (eNOS) expression and reduced plasminogen activator inhibitor-1 (PAI-1) expression. Furthermore, overexpression of KLF2 inhibited the cytokine-mediated induction of tissue factor (TF). In contrast, siRNA-mediated knockdown of KLF2 reduced antithrombotic gene expression while inducing the expression of pro-coagulant factors. The functional importance of KLF2 was verified by in vitro clotting assays. By comparison to control infected cells, KLF2 overexpression increased blood clotting time as well as flow rates under basal and inflammatory conditions. In contrast, siRNA-mediated knockdown of KLF2 reduced blood clotting time and flow rates. These observations identify KLF2 as a novel transcriptional regulator of endothelial thrombotic function. The full text of this article is available online at http://circres.ahajournals.org. (Circ Res. 2005;96:e48-e57.)

Key Words: endothelium • coagulation • thrombomodulin • transcription

One of the central functions of the vascular endothelium is to maintain blood fluidity. Under physiological conditions, this occurs through the production of factors that inhibit blood coagulation (eg, thrombomodulin, TM), inhibit platelet aggregation (eg, nitric oxide, NO), and promote fibrinolysis (eg, tissue plasminogen activator, tPA). Hemostasis may occur if the capacity of inhibitory pathways is impaired or because the capacity of endogenous anticoagulant systems is overwhelmed by a noxious stimulus.

Both biomechanical and biochemical stimuli can affect endothelial gene expression and, as a consequence, its ability to regulate blood fluidity. For example, laminar shear stress (LSS) induces various factors such as endothelial NOS (eNOS) and thrombomodulin (TM) that confer potent antithrombotic and anti-inflammatory properties. In contrast, the endothelial surface loses its nonthrombogenic properties in response to cytokines such as TNF-α or IL-1β and enzymes such as thrombin. By inducing the production of procoagulant factors (eg, tissue factor, TF), inducing the production of antifibrinolytic substances (eg, plasminogen activator inhibitor 1, PAI-1), and inhibiting the expression of natural anticoagulants (eg, thrombomodulin), these noxious stimuli confer prothrombotic properties to the endothelial surface. These observations also highlight the intimate relationship between blood coagulation and inflammation.

Kruppel-like factors (KLFs) are a subclass of the zinc-finger family of transcription factors characterized by the DNA binding domain containing the conserved sequence CX2C3FX5LX2HX3H (X is any amino acid; underlined cysteine and histidine residues coordinate zinc). KLFs typically bind to GC-rich or CACCC sequences in the promoter region of target genes to regulate transcriptional activity. Studies from our laboratory and others demonstrate an emerging role for this family of transcriptional regulators in vascular biology. One member of this family, KLF2, has been identified as a negative regulator of T cell activation and adipogenesis. With respect to the vasculature, KLF2 is expressed exclusively in the endothelium. The importance of KLF2 was verified by gene-targeting experiments demonstrating lethality at embryonic day 12.5 to 14.5 due to abnormal blood vessel architecture. Recently, we and others identified KLF2 as being induced by laminar shear stress and...
inhibited by proinflammatory cytokines.16-22 Our previous study identified KLF2 as a potent inducer of endothelial nitric oxide synthase (eNOS) expression and activity. Furthermore, we demonstrated that KLF2 inhibited the inflammatory cytokine-mediated induction of adhesion molecule expression and immune cell adhesion.16

A role for this factor in endothelial thrombotic function has not been elucidated. Using overexpression and siRNA-mediated knockdown approaches, we provide evidence that KLF2 regulates the expression of key endothelial thrombotic factors under basal and cytokine-stimulated conditions. As a consequence, blood clotting time and flow rates are increased in the presence of KLF2 and reduced by KLF2-deficiency. These observations identify KLF2 as a novel regulator of endothelial thrombotic function.

Materials and Methods

Cell Culture and Reagents
Human umbilical vein endothelial cells (HUVECs) were acquired from Cambrex BioScience and cultured in EBM-2 media according to manufacturer’s instructions. Bovine aortic endothelial cells (BAEcs) were from Cell Applications and maintained in Dulbecco Modified Eagle Medium with 10% FBS and 1% penicillin/streptomycin. In some experiments, HUVECs were treated with human IL-1β, human TNF-α (R&D systems), and thrombin (Sigma) at final concentrations of 2.5 ng/mL, 10 ng/mL, and 2 U/mL, respectively, for 4 hours. TM and von Willebrand factor (vWF) cDNA was generated by RT-PCR; the endogenous human KLF2 mRNA expression was detected using a 3’UTR fragment of the human KLF2 cDNA generated by RT-PCR. eNOS and PAI-1 cDNA were a kind gift from Dr J.K. Liao (Harvard Medical School, Boston, Mass). Exogenous KLF2 mRNA expression was detected using a mouse KLF2 cDNA probe, whereas exogenous protein was detected using a polyclonal antibody (gift from J. Leiden, Abott Laboratories, Chicago, Ill). Antibodies recognizing thrombomodulin and PAI-1 were purchased from Santa Cruz Biotechnology; tissue factor (TF) antibody was from American Diagnostica; eNOS antibody was from BD Biosciences; and α-tubulin antibody was from Sigma. All adenoviral constructs were generated by the Harvard Gene Therapy Initiative.

Northern and Western Blot Analysis
HUVECs were infected with Ad-GFP and Ad-GFP-KLF2 for 24 hours, exposed to the indicated stimulus, and then harvested for total RNA and/or protein analysis. Total RNA was obtained by using Trizol following the manufacturer’s instruction, and Northern blot studies were performed as previously described.23 Cellular protein was extracted in RIPA buffer (Tris-HCl, pH 7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with the Complete protease inhibitor cocktail (Roche), and Western blot analyses performed using the indicated antibodies as previously described.16

Transient Transfection Assays
The 2.2-kb proximal human thrombomodulin promoter was generated by PCR from genomic DNA and cloned into pGL3-Basic vector. The PAI-1 (~800-Luc) promoter was a kind gift of Dr D. Loskutoff ( Scripps Institute, La Jolla, Calif); vWF promoter was obtained from Dr N. Jahroudi (Albert Einstein College of Medicine, New York, NY). Site-specific mutagenesis was accomplished by using the QuikChange mutagenesis kit following the manufacturer’s instruction (Stratagene). HUVECs or BAEcs were plated at a density of 5 × 10^4/well in 12-well plates 1 day before transfection. Transient transfection studies were performed using Fugene 6 reagent (Roche Molecular Biochemicals) according to instructions by the manufacturer. A total of 1 μg plasmid DNA was used in transfections, and total DNA was always kept constant. Cells were harvested 48 hours after transfection at which time they were nearly confluent. Then, assays were performed for luciferase activity and normalized to β-galactosidase activity in each sample. In some experiments, cells were treated with human TNFα (10 ng/mL) for 6 hours before harvest. All transfections were performed in triplicate (n = 6 to 9).

Thrombomodulin and Tissue Factor Activity Assays, PAI-1 Secretion Assay
Both thrombomodulin and tissue factor activity on the surface of HUVECs was measured by a chromogenic assay.23 Endothelial thrombomodulin enzyme activity was measured by the production of activated protein C (APC) from protein C. Briefly, HUVECs were washed with cold TBS (50 mmol/L Tris HCl, 120 mmol/L NaCl, 2.7 mmol/L KCl, and 3 mg/ml bovine serum albumin) three times, and immediately thereafter, cells were incubated with 150 mmol/L human protein C (Enzyme research laboratories) and 0.5U/mL of thrombin for 1 hour at 37°C. The reagents were quenched by adding hirudin (Sigma) and APC activity measured using a chromogenic substrate (S2366, Chromogenix). The reaction was stopped after 10 minutes by the addition of acetic acid and the amidolytic activity of activated protein C generated was read at 405nm with a spectrophotometer. The TM activity was expressed in arbitrary units, using reference curves determined with purified human activated protein C (Enzyme Research Laboratories). For TF activity assay, HUVECs were stimulated with 10 ng/mL of TNF for 4 hours. Subsequently, 5 mmol/L of FVIIa and 150 mmol/L of FX (Enzyme Research Laboratories) were then added and incubated at 37°C for 30 minutes. Substrate S-2765 (Chromogenix) was added and activity measured as mentioned earlier for thrombomodulin. PAI-1 secretion was measured by a kit from American Diagnostica according to the manufacturer’s recommendation.

siRNA Transfection
Human KLF2-directed siRNA (5’-UGUCUGAGGCCCAGC-CAAAUU-3’) and a nonspecific control siRNA (5’-AUGAAGCUGAAUGCUCAAU-3’) were purchased from Dharmacon. HUVECs were plated 1 day before transfection in antibiotic-free EBM-2 medium. On the day of transfection, 100 mmol/L of specific siRNA targeting human KLF2 or nonspecific siRNA was incubated with Lipofectamine 2000 (Invitrogen) at room temperature for 30 minutes before adding to the HUVECs in OPTI-MEM (Invitrogen). Three hours later, the medium was replaced by EBM-2 and cultured for an additional 48 hours. TM activity (Figure 1B) activity was assessed as described above according to manufacturer’s recommendation. For other knockdown studies, cells were treated with or without TNFα for 2 hours and harvested for protein and/or RNA to assess target gene expression (Figure 5B).

In Vitro Blood Flow Assays
HUVECs cultured in 3.5-cm petri cell culture dishes (no. 353001, Becton Dickinson) were infected with Ad-KLF2 virus or Ad-GFP (control adenovirus) at 10 MOI for 24 hours. Infection was estimated visually at ~80% using fluorescence microscopy. For siRNA experiments, cells were transfected for a total of 48 hours before study. On the day of the experiment, one set of cells was treated with TNFα (10 ng/mL, final concentration) for 4 hours before perfusion with blood. A control set of cells was exposed to the vehicle (DMSO) for 4 hours. After this incubation, cells were washed twice with PBS and mounted in a parallel plate flow chamber25 (GlycoTech Corporation). The chamber consists of a flow deck machined from a block of polymethylmethacrylate (PMMA). The petri dish containing the cultured cells forms the bottom plate of the chamber. A flow channel of the desired dimensions was obtained by placing a gasket of the same dimensions between the deck and the bottom plate. The flow decks were held together by means of a tight vacuum seal. Connecting tubing was 1/16-inch inner diameter medical-grade Silastic. Native human blood was collected from healthy drug-free volunteers in accordance with an Illinois Institute of Technology-approved IRB protocol after obtaining informed consent from the
donors and immediately perfused through the flow cell at a wall shear rate of 300 seconds$^{-1}$ (0.6 mL/min). Blood was recirculated through the flow cell using a variable speed roller pump (no. 138761, Fisher Scientific) for 30 minutes or until the flow chamber completely occluded (whichever of the two events occurred first). The time to complete occlusion was recorded. Additionally, the flow rate in the flow circuit was measured once every 5 minutes by measuring the amount of blood released from the outlet end of the flow chamber per minute.

**Gel-Shift Studies**

GST-KLF2 fusion protein was produced as described previously. The experiment was performed as described using the Sp1/KLF binding sites from the thrombomodulin promoter.16 KLF2-GST was incubated with the oligonucleotide probe for 30 minutes, and then run on a polyacrylamide gel. The following sequences were used in the gel-shift assay: oligonucleotides harboring −207 Sp1/KLF site: forward: CCTGTCCGGCCGGCCGAGAACCTTC; reverse: GAGGTTCTCGGGCCGGCCGAGCAGG; forward (mutated): CCTGTCCGGCCAAATTCCGAGAACCTTC; reverse(mutated): GAGGTTCTCGAATTGCGGCGAGG.

**Chromatin Immunoprecipitation Studies**

HUVECs were infected with Ad-KLF2-Flag or control virus (Ad-GFP) for 24 hours, and native protein-DNA complexes were cross-linked by treatment with 1% formaldehyde for 15 minutes. The ChIP assay was performed as reported earlier. Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with a mouse anti-flag antibody, or mouse IgG control. The DNA associated with specific immunoprecipitates or with control IgG was analyzed using the following primers: 5′-primer, GAGCACAGAGCCTCGCCTGTCGGCCAAATTCGAGAACCTCC; reverse(mutated): GGAGGTTCTCGGGCGG GGCCGACAGG; forward (mutated): CCTGTCCGGCCAAATTCCGAGAACCTTC; reverse(mutated): GAGGTTCTCGAATTGCGGCGAGG.

**Results**

**KLF2 Induces Thrombomodulin Expression and Activity**

We recently reported the observation that KLF2 is a potent inducer of endothelial nitric oxide synthase, a key regulator of vessel biology.16 To gain greater insight into additional targets of KLF2 in endothelial cells, transcriptional profiling studies were performed using mRNA from HUVECs adenovirally overexpressing KLF2 or a control virus (K. Parmar and G. Garcia-Cardena, unpublished observation, 2004). One of the most potent induced factors was TM, a key regulator of the thrombin-mediated activation of the natural anticoagulant APC. As shown in Figure 1A, adenoviral overexpression of KLF2 (Ad-KLF2-GFP) in HUVECs strongly induced TM mRNA and protein. Consistent with our previous study, eNOS mRNA and protein levels were also strongly increased. To determine whether the increase in TM expression translates into an increase in function, we assessed for enzymatic activity as measured by the production of APC. As shown in Figure 1B, endothelial cells overexpressing KLF2 exhibited an ≈7.7-fold increase in TM activity relative to control adenovirus (Ad-GFP) infected cells. These data demonstrate that KLF2 over-expression robustly increases thrombomodulin expression and activity. We then performed siRNA-mediated KLF2 knockdown to assess the effect of KLF2 deficiency on endogenous TM and eNOS protein levels. Because a specific antibody to human KLF2 is lacking, a parallel set of experiments were performed and harvested for total RNA and protein. As shown in Figure 1C, in contrast to nonspecific siRNA (NS; Figure 1C), KLF2 mRNA expression is strongly reduced in the presence of a specific siRNA in HUVECs. Furthermore, knockdown of KLF2 resulted in a marked reduction in basal TM and eNOS protein levels (Figure 1C, top right). The reduction in TM correlated with an approximately 68% reduction in activity (Figure 1B, bottom). These data identify KLF2 as one of the transcription factors that regulates TM and eNOS expression in endothelial cells.

**KLF2 Regulates the Thrombomodulin Promoter**

KLFs are transcription factors that bind DNA and regulate gene expression. Previous studies demonstrate that KLFs can bind to sequences such as 5′-CACC-3′, 5′-GTGGG-3′ (GT-box), or GC-rich sequences such as Sp1/3 sites (Sp/KLF sites).16,19,20 To determine the molecular basis of the ability of KLF2 to induce thrombomodulin expression, we assessed its effect on thrombomodulin promoter activity. A −2.2-kB fragment of the human thrombomodulin promoter was cloned and used for subsequent studies. As shown in Figure 2A, KLF2 strongly induced the thrombomodulin promoter in HUVECs. This effect was specific as one other family members (KLF5) was unable to transactivate the thrombomodulin promoter (Figure 2A). Furthermore, this induction required an intact KLF2 because neither the DNA-binding domain alone (ZnF) or non-DNA-binding domain (KLF2ΔZnF) was able to induce promoter activity (Figure 2B).

Examination of the thrombomodulin promoter revealed the presence of numerous potential binding sites. To determine the region within the TM promoter responsible for KLF2 mediated transactivation, a series of deletion and mutational analyses were undertaken. As shown in Figure 2C, KLF2 is able to transactivate the −2.2kb-Luc approximately 27-fold. Deletion studies indicate that the majority of activity is retained within the −0.385kB-Luc fragment. However, KLF2 mediated induction is essentially lost with the −0.155kb-Luc promoter construct (Figure 2C). These studies localize the KLF2 mediated induction to the region between −0.385 to −0.155 kb.

Close examination of this critical 231-bp region revealed the presence of two KLF and two Sp/KLF sites. Importantly, the first Sp1/KLF site (labeled Sp/KLF1) has been previously shown as critical for basal TM promoter activity in endothelial cells.32 To determine the importance of these sites, promoter mutation studies were undertaken. Mutation of the two KLF sites, individually or in combination, did not alter the ability of KLF2 to transactivate the −0.385-kb Luc promoter construct (Figure 2D and data not shown). In addition, mutation of the Sp/KLF 2 site resulted in a mild reduction (data not shown), whereas mutation of the Sp/KLF1 site eliminated the majority of KLF2’s inductive effect (Figure 2D). These data suggest that the Sp/KLF1 site mediated induction of the TM promoter by KLF2.

To verify that KLF2 can bind to this site, gel-shift studies and chromatin immunoprecipitation (ChIP) studies were undertaken. As shown in Figure 2E, robust binding was seen with GST-KLF2 (but not GST-alone; data not shown) to this site and the specificity of binding verified by competition...
studies. Furthermore, as shown in Figure 2F, ChIP studies verified the ability of adenovirally overexpressed KLF2 to bind to the TM promoter.

KLF2 Inhibits Cytokine Induced Prothrombotic Gene Expression

Proinflammatory stimuli are known to confer procoagulant properties to endothelial cells. For example, cytokines such as IL-1β and TNFα or the enzyme thrombin can induce expression of procoagulant factors such as TF and PAI-1. In a previous report, we found that IL-1β treatment of HUVECs reduced KLF2 expression, and that forced overexpression of KLF2 prevented the cytokine-mediated induction of adhesion molecules such as E-selectin and VCAM-1. As such, we hypothesized that KLF2 may inhibit cytokine mediated induction of procoagulant factors.

For these studies, we used TNF-α as a stimulus. Here, we found that TNF-α treatment of HUVECs strongly reduced endogenous KLF2 expression (Figure 3A). A similar effect was seen with thrombin and IL-1β. To assess the effect of KLF2 overexpression on cytokine-mediated regulation of procoagulant gene expression, we adenovirally overexpressed KLF2 or control virus in the presence or absence of TNF-α. Consistent with our previous publication, KLF2 induced eNOS and inhibited the TNF-α mediated induction of E-selectin. We then assessed the expression of several key factors involved in blood coagulation. As shown in Figure 3B, under basal conditions, KLF2 strongly induced thrombomodulin and inhibited vWF and PAI-1. A mild but reproducible increase was noted in the expression of tPA. Furthermore, the cytokine-mediated induction of TF and PAI-1 was potentiy attenuated, whereas the cytokine-mediated inhibition of TM was prevented (Figure 3B). Finally, in agreement with the reduction in endogenous mRNA expression, transient transfection studies demonstrate that KLF2 inhibits the PAI-1, vWF, and TF promoters under basal conditions and/or after TNF-α stimulation (Figure 3C).
To verify that the effects seen at the mRNA level translate into similar effects at the protein level, Western blot studies were performed. As shown in Figure 4A, under basal conditions, KLF2 overexpression strongly induced eNOS and TM protein while inhibiting PAI-1 levels. Furthermore, the TNF-α-mediated induction of TF and PAI-1 protein was strongly inhibited. We also assessed for PAI-1 antigen secretion and TF activity. As shown in Figure 4B (left), KLF2 inhibited both basal and TNF-α-induced PAI-1 protein. Furthermore, the induction of TF activity by TNFα, as assessed by the generation of Factor Xa, was strongly inhibited by KLF2 (Figure 4B, right). Taken together, these data indicate that forced expression of KLF2 prevents the TNF-α-mediated effects on procoagulant gene expression.

KLF2 Increases Clotting Time and Flow Rates Under Basal Conditions and in Response to TNF-α
To determine the functional consequence of the effect of KLF2 on blood clotting, in vitro blood clotting assays were performed. HUVECs grown on culture dish were infected with Ad-GFP or Ad-KLF2 for 24 hours and treated with TNF-α for 4 hours. Native human whole blood was perfused over the cultured, infected, and stimulated cells and blood flow...
rate as well as occlusion times were recorded. On exposure to control cells (HUVECs infected with control Ad-GFP), native blood clotted and completely occluded the flow system within 20.3 ± 0.57 minutes of perfusion (Figure 4C; top). In contrast, perfusion of blood over KLF2 overexpressing HUVECs significantly increased clotting time to 29.7 ± 0.57 minutes. The stimulation of cells with TNF-α shortened occlusion times in both the control as well as KLF2 group. In this case too, as shown in Figure 4C (top), time to reach occlusion in the KLF2 infected HUVECs (20.7 ± 3.8 minutes) was significantly longer than control group (14.7 ± 0.57 minutes). Furthermore, flow rates measured every 5 minutes at the outlet end of the flow chamber demonstrated that KLF2 was able to decelerate blood clotting if not inhibit complete occlusion. As shown in Figure 4C (bottom), either in the presence or absence of TNF-α, the flow rate in the flow circuit containing the KLF2-infected HUVECs was significantly higher than that in control group (which reached 0 on occlusion).

Effect of KLF2 Knockdown on Coagulant Gene Expression
The studies above and the attendant conclusions are largely derived from overexpression studies. To determine the importance of KLF2 in endothelial coagulant gene expression under basal condition or in the presence of TNF-α, siRNA-mediated knockdown studies were undertaken. For these studies, the TNF-α stimulation was for 2 hours in an effort to determine whether siRNA-mediated knockdown of KLF2 accelerated the cytokine mediated induction of key procoagulant substances such as TF. As shown in Figure 5, knockdown of KLF2 (Figure 5A) resulted in a strong reduction in eNOS and TM (80% reduction; Figure 5B). Furthermore, in the presence of TNF-α, the induction of TF is enhanced by KLF2 deficiency. A mild effect was seen on PAI-1 levels.

To determine whether a reduction in KLF2 levels accelerated blood clotting time, we performed in vitro clotting assays. Under basal conditions (Figure 5C, upper graph), siRNA mediated knockdown of KLF2 reduced blood clotting time from 19.3 ± 0.60 minutes (labeled NS) to 13.3 ± 0.57 minutes (labeled siKLF2). After TNF-α stimulation, KLF2 deficiency reduced blood clotting time from 14 ± 1.0 minutes (labeled NS) to 10.3 ± 0.58 minutes (labeled siKLF2). Furthermore, as shown in Figure 5C (bottom graph), either in the presence or absence of TNFα, the flow rate in the flow circuit containing siKLF2 HUVECs was slower than that of the control group.

Discussion
The vascular endothelium maintains blood fluidity by producing inhibitors of coagulation and platelet aggregation,
modulating vascular tone, and providing a protective barrier separating hemostatic blood components from subendothelial structures. Previous studies from our laboratory identified KLF2 as a novel and potent regulator of eNOS and proinflammatory activation of the endothelium. In this report, we build on these initial observations and provide evidence that KLF2 regulates endothelial coagulant gene expression and thrombotic function.

The vascular endothelial cells produce a number of anticoagulant substances that, under physiological states, maintain blood fluidity. TM was originally identified in endothelial cells as a cell surface cofactor that increases the rate of thrombin-catalyzed protein C activation. The activation of protein C (PC) is further amplified when it is bound to EPCR. Recent studies suggest that the TM impacts not only coagulation but also on inflammation, fibrinolysis, and cellular proliferation. Despite the importance of this factor in multiple aspects of endothelial biology, the molecular mechanisms regulating TM expression remain poorly understood. In this regard, our studies provide important insights. We
found that KLF2 overexpression robustly induces TM mRNA, protein, and activity (Figure 1A and 1B). Furthermore, siRNA-mediated knockdown of KLF2 results in a marked reduction in basal TM expression and activity (Figure 1B and 1C). Taken together, these data strongly implicate KLF2 as a novel regulator of TM expression in ECs.

Our studies also provide insight into the transcriptional basis for the ability of KLF2 to induce TM expression. Studies to date suggest that an Ets-binding site in the proximal promoter and several GC-rich sites (termed Sp1 sites) are important for TM promoter activity.32,44 Furthermore, the retinoic acid receptors (RARα, RARβ, and RARγ) have been shown to increase TM promoter activity.45 The KLFs and Sp1 factors together constitute a subgroup within the larger family zinc-finger transcription factors.46 Consistent with their close relationship, both KLFs and Sp1 proteins can bind to similar DNA sequences such as GC-rich sequences, 5'-CACCC-3' and 5'-GTGGG-3' sequences. Furthermore, with respect to KLF2 specifically, a recent study demonstrated that KLF2 can bind to and transactivate the p21 promoter via a Sp1–3 site.31 These considerations are highly relevant in light of our promoter deletion studies. Inspection of the 2.2-kb TM promoter revealed the presence of numerous KLF or Sp/KLF sites. Promoter deletion studies localized a region between 0.385 and 0.155 kb as requisite for the ability of KLF2 to transactivate this promoter. These
results are consistent with previous reports that identify Sp1 sites within this region as important for maximal TM promoter activity in HUVECs. Single and combinatorial mutational analyses within this 231-bp region (Figure 2D) coupled with gel-shift and ChIP assays implicate the −207bp Sp/KLF site as critical for the inductive effects of KLF2 (Figure 2E and 2F). A limitation of our ChIP study is that it relies on the overexpression of a tagged KLF2 construct because a suitable antibody for endogenous KLF2 for this type of assay is lacking. However, despite this limitation, our data do support a role for KLF2 in regulating TM promoter activity.

Our studies also address the role of KLF2 in the context of the intimate relationship between inflammation and coagulation. Inflammatory mechanisms induce procoagulant factors and inhibit the expression of natural anticoagulants as well as fibrinolytic activity. Among the key effects of inflammatory cytokines are a reduction in TM expression, and induction of tissue factor and PAI-1. Previous studies from our laboratory identified KLF2 as a potent inhibitor of NF-κB activity principally through the recruitment of key coactivators such as p300/CPB. As such, we reasoned that KLF2 may also inhibit the prothrombotic effects of inflammatory cytokines. Indeed, as shown in Figure 3, KLF2 overexpression strongly inhibited the TNF-α-mediated induction of TF and PAI-1 while preventing the inhibition of TM and eNOS. Consistent with the differential regulation of gene expression, ex vivo coagulation studies (Figure 4C) reveal that KLF2 increases both clotting time and flow rates under basal conditions and after treatment with TNF-α. The importance of KLF2 as a regulator of the cytokine-mediated induction of coagulant gene expression is further substantiated by siRNA knockdown experiments. As shown in Figure 5B, knockdown of KLF2 results in a reduction of eNOS and TM expression but an increase in TF expression. Consistent with these effects, blood clotting time and flow rates are reduced by KLF2 deficiency (Figure 5C). These data strongly implicate KLF2 as a key regulator of endothelial coagulant gene expression.

The ability of a single transcriptional regulator to differentially modulate key molecules such as TM, eNOS, PAI-1, and TF identify that KLF2 as an important regulator of endothelial coagulant function. Furthermore, given the pleiotropic effects of these factors in many physiological and pathological states, the regulatory effect of KLF2 may have implications well beyond coagulation. Finally, the observations herein coupled with our previous work implicate KLF2 as a “molecular switch” that regulates important aspects of vascular function in health and disease.

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